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Developmental Potential of Rat L6 Myoblasts *in Vivo* Following Injection into Regenerating Muscles

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To examine the relative importance of myoblast lineage and environmental influences on the development of muscle fiber types *in vivo*, the phenotype of muscle fibers formed from rat L6 myoblasts was examined following their injection into different regenerating adult muscles. Myoblasts were infected with a retroviral vector carrying a LacZ reporter gene and their fate *in vivo* was examined using a panel of antibodies against various myosin heavy chain (MyHC) isoforms. Since L6 myoblasts express IIX MyHC following differentiation *in vitro*, we wanted to determine if they would form IIX muscle fibers *in vivo* and whether innervation would alter this fate. Following injection, L6 cells either fused with each other to form homotypic fibers or fused with host muscle cells to form heterotypic fibers. Initially, homotypic fibers expressed embryonic MyHC—similar to L6 myotubes *in vitro*. However, by 4 weeks postinjection IIX MyHC had replaced embryonic MyHC as the predominant isoform. Single fiber analysis using an antibody specific for NCAM indicated that this transition was independent of innervation. Analysis of heterotypic fibers resulting from the incorporation of donor L6 myoblasts into host fast IIA and IIB fibers revealed that L6-derived nuclei express embryonic and IIX MyHCs for up to 8 weeks postinjection, often as nuclear domains surrounding L6 nuclei. These results suggest that MyHC expression in muscle fibers derived from L6 myoblasts is regulated, in part, by intrinsic factors that limit the fiber type potential of these cells *in vivo*. © 1997 Academic Press

INTRODUCTION

Adult mammalian muscle consists of several different fast fiber types (IIA, IIB, and IIX) and one slow fiber type (I), all of which can be characterized by differences in their speed of contraction (Schiaffino and Reggiani, 1996), resistance to fatigue (Gauthier, 1986), and pattern of myosin heavy chain (MyHC) expression (Armstrong and Phelps, 1984). In addition to the adult fast (IIA, IIB, IIX) and slow (type I) MyHC isoforms there are also several developmental isoforms, including embryonic and neonatal MyHCs, which are only expressed during muscle development (Condon *et al.*, 1990; Hughes *et al.*, 1993) and muscle regeneration (Whalen *et al.*, 1990). The expression of the various MyHCs, including the down-regulation of the developmental isoforms, has been studied extensively in order to obtain insight into the mechanisms which regulate the development of the various muscle fiber types.

Cross-innervation studies in which adult slow muscles were denervated and reinnervated by fast motoneurons demonstrated a transition in the muscle phenotype from slow to fast (Buller *et al.*, 1960). This suggested that the type of innervation received by the muscle governed the final fiber phenotype. Experiments causing changes in the electrical stimulation patterns of the muscle also illicited similar switches in MyHC expression (reviewed by Pette and Vrbova, 1992), indicating that the changes caused by the nerve were due to the frequency of stimulation rather than specific trophic factors. Interestingly, coculturing of spinal cord and muscle produced the up-regulation of adult fast isoforms not normally present in cultured myotubes, even in the absence of synapse formation (Ecob-Prince *et al.*, 1986). Therefore, direct innervation may not be necessary for the up-regulation of the fast isoforms. These studies suggested that the environment regulates the pattern of MyHC expression in mature muscle fibers, with the nerve involved in the overall modification of that pattern.

Recently, a growing body of evidence has suggested that

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intrinsic factors may also be involved in dictating the final phenotype of muscle. Several myoblast cell lines have been shown to express adult fast and slow isoforms without the presence of trophic or electrical stimuli from the nerve [Cox et al., 1993; MacIntyre and Merrifield, 1997]. As well, examination of cultured myoblasts obtained from developing hindlimbs revealed the presence of different patterns of MyHC expression, depending on the time of gestation at which the myoblasts were obtained. In mice [Smith and Miller, 1992; Vivarelli et al., 1988], quail [Miller et al., 1986], and rats [Pin and Merrifield, 1993], myoblasts cultured from embryonic hindlimb buds accumulated predominantly slow MyHC, while myoblasts cultured from later stages of development expressed fast MyHC isoforms in addition to developmental MyHCs. Analysis of normal muscle development has also revealed two populations of myotubes that appear at different time points in gestation [Ross et al., 1987], which may result from the fusion of the different myoblast lineages observed *in vitro*. Characterization of these two myotube populations supported this conclusion, since the early population of primary myotubes exhibited a typical slow phenotype while the later population of secondary myotubes exhibited fast patterns of MyHC expression [Condon et al., 1990]. In rats, adult fast MyHC is absent from primary myotubes while low amounts of adult fast MyHCs have been detected in secondary myotubes almost immediately after their inception [Cho et al., 1994]. These findings suggest that intrinsic factors related to the developmental origins of different myoblast lineages may establish patterns of MyHC expression prior to innervation and maturation.

To examine the effects of extrinsic factors on the phenotype of separate myoblast populations, investigators have transplanted characterized myoblast populations into various muscle environments [Hughes and Blau, 1992; DiMario et al., 1993]. Injection of quail embryonic myoblasts or satellite cells into developing chicken hindlimbs produced predominantly donor-derived myotubes. The pattern of MyHC expression in these homotypic myotubes was similar to that observed *in vitro* [DiMario et al., 1993; DiMario and Strockdale, 1995]. However, these investigators did not determine if the homotypic myotubes generated under these conditions became innervated. In addition, the developmental potential of these myoblasts could only be followed for 10 days, preventing the analysis of long term environmental effects on this phenotype. In a different study, injection of C_2C_{12} myoblasts or mouse satellite cells into the muscles of adult mice resulted in an alteration of the MyHC phenotype found *in vitro* when injected myoblasts fused with host muscle cells to form heterotypic fibers. The majority of MyHC isoforms found in culture were down-regulated with maintenance of only one isoform typical of the muscle fibers into which these myoblasts were incorporated. These results supported the view that innervation ultimately controls muscle phenotype *in vivo* [Hughes and Blau, 1992]. Unfortunately, predominantly donor-derived fibers were not observed in this study and all of the labeled fibers appeared to be the result of the fusion of a small number of donor myoblasts with a substantially larger number of muscle fibers.

Since these two studies differed greatly in their design and in the type of fibers analyzed, it has not been possible to clearly define the relative contribution of intrinsic and extrinsic influences on the development of muscle fiber phenotypes *in vivo*. To address this problem, we have injected rat L6 myoblasts into regenerating hindlimb muscles of adult rats. The rationale for using L6 myoblasts is that this line expresses only two MyHCs *in vitro*—embryonic fast and adult IIX MyHC—and may be committed to forming IIX muscle fibers *in vivo* [Wieczorek et al., 1985; Pin and Merrifield, 1997]. The injection of these myoblasts with marcaine to induce muscle degeneration/regeneration also offered some advantages. First, with the degeneration of muscle tissue induced by marcaine, the cells would be introduced into an area actively promoting proliferation and differentiation [Bischoff, 1986]. Consequently, the cells would have the option of fusing with each other or with host satellite cells and fibers. Second, the denervation and muscle degeneration should induce axon sprouting [Dabrowska and Landmesser, 1988] and increase the likelihood that predominantly donor-derived myotubes would become innervated. The innervation of such myotubes, starting at 4 weeks postinjection, has previously been observed in experiments where large scale degeneration occurred prior to myoblast transplantation [Wernig et al., 1991]. The innervation status of individual fibers can be assessed by examining the expression of neural cell adhesion molecules (NCAM) along the surface of the fiber, since NCAM is localized along the entire length of nascent muscle fibers but becomes localized exclusively to the motor endplate following innervation [Covault and Sanes, 1985; Covault et al., 1986].

In this study, L6 myoblasts infected with a constitutively expressed LacZ reporter gene were injected into different muscle groups and their developmental potential was examined immunohistochemically over an 8-week period using a panel of antibodies specific for the different MyHC isoforms. Our results demonstrate that homotypic muscle fibers derived predominantly from L6 myoblasts maintain their *in vitro* pattern of MyHC expression, since they accumulate detectable levels of only embryonic and IIX MyHCs. Interestingly, there is a transition in the phenotype of these myotubes in that the embryonic MyHC disappears starting at 28 days postinjection and is eventually replaced by IIX MyHC. However, no correlation between innervation and MyHC expression was observed. When heterotypic fibers were examined in the superficial region of tibialis anterior muscle (which contains a large proportion of type IIB fibers) and in the lateral gastrocnemius muscle (which contains a

TABLE 1
Myosin Heavy Chain Specificity of Monoclonal Antibodies

Monoclonal antibody[a]	MyHC specificity	Isotype	Dilution indirect	Dilution ABC
47A[1]	Embryonic	IgG_{2A}	1:10	1:10
NN6[2]	Neonatal	Rabbit polyclonal	1:40	1:350
MY-32[3]	Neonatal, adult fast	IgG_1	1:100	1:200
212F[4]	IIB, IIX	IgG_{2b}	1:3	1:4
4A.74[3]	IIA	IgG_1	Undiluted	Undiluted
SC.71[4]	IIA	IgG_1	Undiluted	Undiluted
BF.F3[5]	IIB	IgM	Undiluted	Undiluted
BF.35[5]	All except emb. and IIX	IgG_1	1:50	1:50
8H8[6]	Slow	IgG_{2A}	1:5	1:5
10D10[7]	Slow	IgG_{2A}		

[a] Antibodies obtained from (1) Pin and Merrifield (1993), (2) Barker-Browne et al. (1984), (3) Hughes et al. (1993), (4) Sigma, (5) Denardi et al. (1993), (6) Bourrageat et al. (1984). See text for details.

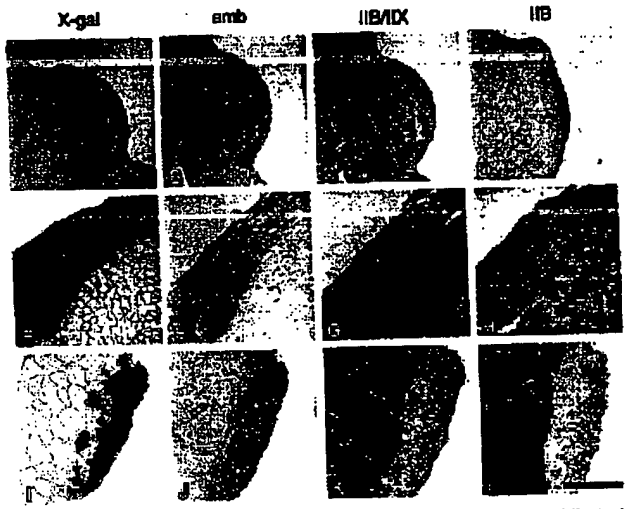


FIG. 1. Pattern of myosin heavy chain (MyHC) expression in homotypic muscle fibers at 1, 4, and 8 weeks following injection of L6BAG-A4 myoblasts into the plantaris muscle. Serial sections were either reacted with X-gal substrate (A, E, I) or analyzed for MyHC expression using immunohistochemistry with Mabs specific for embryonic (47A; B, F, J), fast IIB/IIX (212F; C, G, K), or fast IIB MyHCs (BF.F3; D, H, L). At 1 week postinjection (PI; A–D), X-gal staining reveals the presence of darkly staining, predominantly donor-derived myotubes at the periphery of the muscle (A, *). These myotubes react strongly for embryonic MyHC (47A; B). Analysis with Mabs specific for IIB/IIX MyHC (212F; C) and IIB MyHC (BF.F3; D) or other MyHCs (not shown) did not react with the injected cells, suggesting that embryonic MyHC is the predominant isoform expressed in the homotypic fibers at this time. At 4 weeks PI (E–H), most of the peripherally located, X-gal positive fibers (E, *) react with Mabs against embryonic (F), and IIB/IIX (G) MyHC but not IIB MyHC (H), suggesting that both embryonic and IIX MyHC are being coexpressed in homotypic fibers. At 8 weeks PI (I–L), homotypic fibers react strongly for IIB/IIX MyHC (K), but not for IIB MyHC (L). Reaction for embryonic MyHC is relatively weak (J), suggesting that IIX MyHC is now the predominant isoform being expressed. The degree of reaction in homotypic fibers was judged relative to the fiber-type specific reactions in the adjacent host muscle in each section. The dark spots in the injection sites are from the India ink injected with the L6BAG-A4 myoblasts. Bar, 370 μm in A–D, 270 μm in E–H, and 180 μm in I–L.

TABLE 2
Myosin Heavy Chain expression of Homotypic L6BAG-A4 Myotubes

Weeks after injection	Muscle	Myosin heavy chain expression: Embryonic	Neonatal	IIA	IIB	IIX	Slow
1	Gastroc.	+++	–	–	–	–	–
	Soleus	+++	–	–	–	+	–
	Plantaris	+++	–	–	–	–	–
	TA	+++	–	–	–	–	–
2	Gastroc.	+++	–	–	–	+	–
	Soleus	+++	–	–	–	+	–
	Plantaris	+++	–	–	–	+	–
	TA	+++	–	–	–	+	–
4	Gastroc.	++	–	–	–	++	–
	Soleus	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Plantaris	+++	–	–	–	+++	–
	TA	++	+	–	–	+++	–
8	Gastroc.	++	–	–	–	+++	–
	Soleus	++	n.d.	–	n.d.	++	–
	Plantaris	++	+	–	–	+++	–
	TA	++	+	–	–	+++	–

Note. +++, >90% of myotubes are positive; ++, <90% of myotubes are positive; +, <5% of myotubes are positive. n.d., not determined.

mixture of different type II fibers) (Armstrong and Phelps, 1984), the expression of IIX MyHC was maintained, often in conjunction with the MyHC isoforms characteristic of the host fiber. However, in heterotypic fibers resulting from the incorporation of L6 myoblast nuclei into slow type I fibers, IIX MyHC was only transiently expressed. These results suggest that MyHC expression in muscle fibers formed from L6 myoblasts is regulated, in part, by intrinsic factors that limit the fiber type potential of these cells *in vivo*.

MATERIALS AND METHODS

Infection of L6 Rat Myoblasts with a β-Galactosidase Reporter Gene

A subclone of the L6 rat myoblast cell line originally isolated by Yaffe (1968) was obtained from Dr. B. D. Sanwal (Department of Biochemistry, University of Western Ontario), and grown in complete alpha-minimal essential medium (α-MEM) containing 10% horse serum and 50 μg/ml of gentamycin as previously described (Clarke *et al.*, 1989). The ecotrophic retrovirus producer cell line PSI 2 BAG α was obtained from the American Type Culture Collection (Bethesda, MA) and grown in complete Dulbecco's Modified Eagles Medium (DMEM), 4.5 g/liter glucose, 10% fetal calf serum, 10 m*M* penicillin, 5 m*M* streptomycin, and 50 μg/ml fungizone. Viruses (BAG) produced by this line contain the lacZ gene under control of the LTR promoter and the transposon Tn5 neomycin-resistance gene (neo^R) under control of the SV40 early promoter (Price *et al.*, 1987). To produce transducing BAG virus, PSI 2 BAG α cells were grown for 1 day postconfluence in T75 flasks, refed with complete DMEM medium and incubated for another 48 hr. Conditioned medium containing the BAG retrovirus was harvested, centrifuged at 800*g* × 10 min, and filtered through a 0.45-μ*M* filter to remove cells and stored as 10-ml sterile aliquots at −80°C.

To infect L6 myoblasts, log phase cells (10^6/T75 flask) were incubated with 15 ml of complete α-MEM medium containing 4 μg/ml Polybrene (hexadimethrine bromide) and 10% conditioned BAG cell supernatant for three 8-hr intervals, with fresh virus applied at the beginning of each interval. After infection, myoblasts were refed with fresh α-MEM medium and allowed to recover for at least 24 hr before being passaged. After passage, infected cells were grown in α-MEM medium containing 2 mg/ml of the neomycin analog G418 Sulfate (Geneticin) for 2 weeks to kill uninfected cells, then passaged and grown at clonal density in regular medium. To assay for β-galactosidase activity, cultures were fixed with 2% formaldehyde, 0.4% glutaraldehyde in phosphate-buffered saline (PBS), and incubated overnight in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) substrate containing 1 m*M* $MgCl_2$, 10 m*M* potassium ferricyanide, 200 μg/ml X-gal, 150 m*M* NaCl, and 0.1 *M* sodium phosphate, pH 7.5, for 18 hr at 37°C, as described by Shimohama *et al.* (1989). Clones were isolated using cloning cylinders, expanded, and duplicate wells were tested for β-galactosidase (β-gal) activity. Clones in which all cells expressed high levels of β-gal before and after fusion were tested for their ability to fuse normally then expanded for injection into regenerating adult rat muscles.

Injection of L6 Myoblasts into Regenerating Adult Muscles

Once stable clones of L6 myoblasts were obtained in which a high, constitutive level of β-gal could be observed, cells were expanded to obtain large populations for injection. One such clone, L6BAG-A4 myoblasts, was grown to approximately 50% confluence on 100-mm culture dishes, rinsed once with Ca^{2+}, Mg^{2+} free Hanks Balanced Salt Solution (CMF-HBSS), then trypsinized with a 1 in 10 dilution of 2.5% trypsin in CMF-HBSS until all of the cells lifted off the plate. The harvested cells were then collected and washed twice with CMF-HBSS. The resulting pellet was resuspended in a marcaine cocktail containing 0.5% marcaine (bupivacaine hydrochloride), 30 μg/ml (30 units/ml) hyaluronidase, and 0.03% India ink, at a concentration of 1.0×10^6 cells/50 μl. Twenty-seven 2- to 3-month-old rats were anaesthetized using a combina-

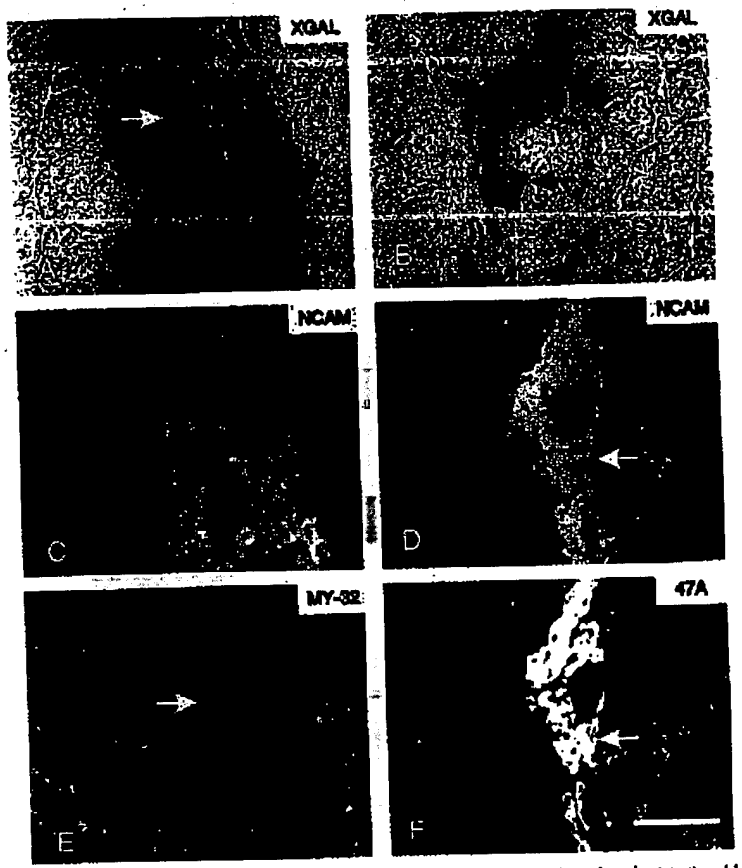


FIG. 2. Immunofluorescent localization of NCAM and MyHC expression in homotypic myotubes 7 days after injection of L6BAG-A4 myoblasts into the extensor digitorum longus muscle of adult rats. Serial sections were either stained for X-gal (A, B) or analyzed using immunofluorescence with an NCAM-specific polyclonal antibody (C, D) or Mabs specific for neonatal/adult fast (E, MY-32) or embryonic (F, 47A) MyHCs. Primary antibodies were identified by fluorescein (NCAM and 47A) or rhodamine-conjugated (MY-32) secondary antibodies. Individual homotypic myotubes (—), determined by their X-gal expression (A, B), are all NCAM positive (C, D), indicating that they are not innervated. These fibers express embryonic MyHC (F) but not fast (IX (E) MyHC at this time. Bar, 65 μm.

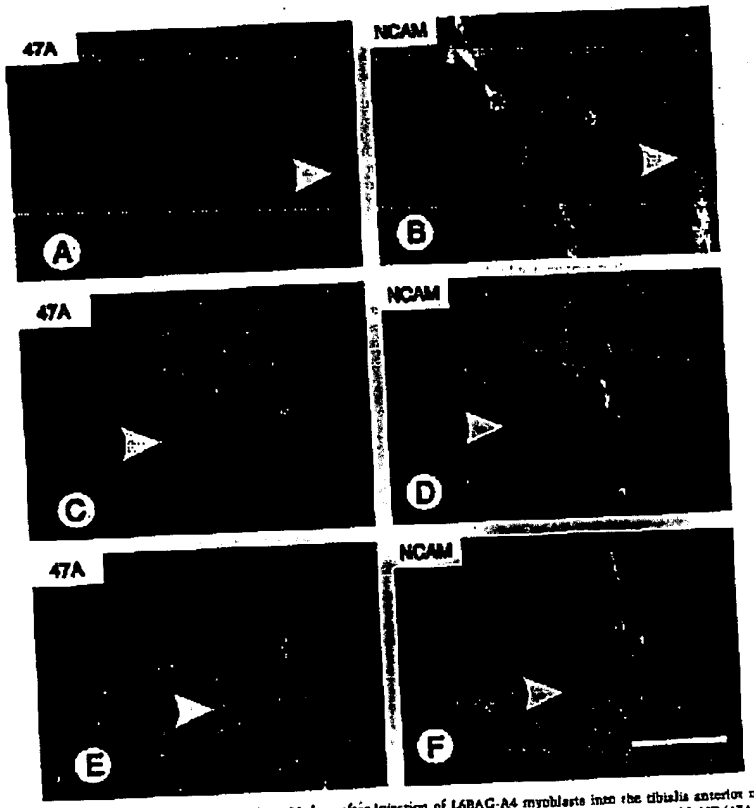
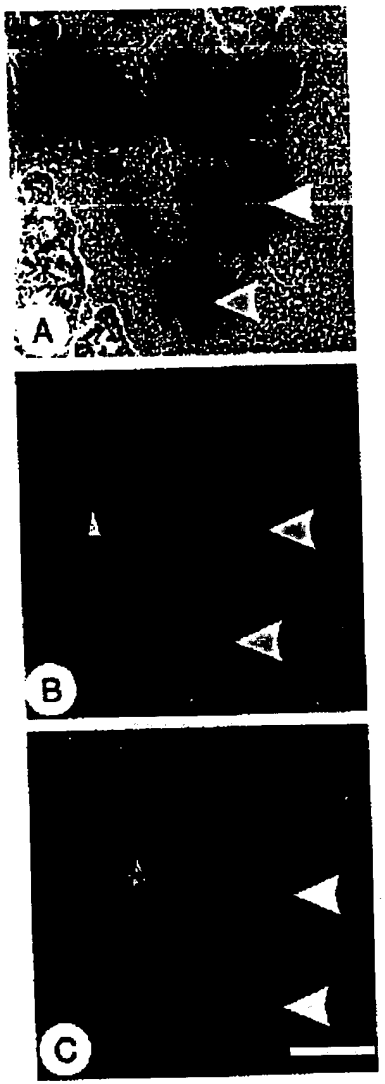


FIG. 3. NCAM expression in homotypic fibers 56 days after injection of L6BAG-A4 myoblasts into the tibialis anterior muscle of adult Wistar Furth rats. Immunofluorescent colocalizations were carried out with antibodies against embryonic MyHC (47A; A, C, E) and NCAM (B, D, F). Primary antibodies were recognized by secondary antibodies conjugated to fluorescein (NCAM) or rhodamine (47A). Myotubes that express NCAM but not embryonic MyHC can be seen in A and B, while myotubes that stain for embryonic MyHC but not NCAM can be seen in C and D, and E and F. All myotubes of interest are labeled (▶). The lack of coordination between the expression of embryonic MyHC and NCAM strongly suggests that innervation is not involved in the downregulation of embryonic MyHC. Bar, 75 μm.

tion of sodium pentobarbital and chloral hydrate. Their hindlimbs were then shaved and swabbed with alcohol prior to injection.

Approximately one million cells in 50 μl of the cocktail were injected into each of three sites—(a) the soleus and plantaris, (b) the gastrocnemius, and (c) the tibialis anterior and extensor digitorum longus muscles of the right leg. Control injections, containing the marcaine cocktail but without cells, were performed in the same manner on the left legs. A 1-ml syringe with a 26-gauge needle was used to perform the injections. The animals were allowed to recover under heat lamps and then maintained as an isolated colony. Each rat initially received a daily injection of cyclosporin A (Sandoz Canada Inc., Montreal, Que) at a concentration of 15 mg/kg starting



body weight. After 4 weeks, cyclosporin injections were reduced to 6 of 7 days to reduce trauma on the rat. Six rats were sacrificed at 7, 14, and 28 days, 4 rats were sacrificed at 42 and 56 days, and one rat was sacrificed at 91 days after injection. Injection sites identified by India Ink were then frozen in melting isopentane, embedded in Tissue Tek OCT freezing compound and serial sectioned at 10–15 μM on a Leitz cryostat. Every tenth section was fixed in 2% formaldehyde, 0.4% glutaraldehyde in PBS, and analyzed for β-gal expression using X-gal substrate as previously described for cultured cells.

Immunohistochemical Analysis of MyHC Expression in Muscle Fibers Containing Donor L6 Nuclei

Sections were examined for MyHC expression using avidin–biotin complex (ABC)-fluorescence or ABC-alkaline phosphatase immunohistochemistry. Sections were blocked in 10% goat serum in phosphate buffered saline (PBS) for 30 min at 37°C, incubated in primary antibody for 1 hr, and washed with PBS. Primary monoclonal antibodies 47A, MY-32, 212F, 4A.74, SC.71, BF.F3, BF.35, 8H8, and 10D10 were used for these analyses. In addition, rabbit polyclonal antibody NN6 was used, which specifically recognizes neonatal MyHC (Butler-Browne and Whalen, 1984). The specificity, source, and optimal dilutions of these antibodies is summarized in Table 1. Sections were then incubated with a secondary antibody, a 1:1000 dilution of either biotinylated goat anti-rabbit (GAR) IgG (against the NN6 antibody) or rabbit anti-mouse (RAM) (gG2A against the 47A antibody) for 1 hr (GAR (gG, Tago Inc, Burlingame, CA; RAM (gG, ICN Biomedicals, Mississauga, Ontario). After the PBS washes, a rhodamine-conjugated avidin complex (at a dilution of 1 in 50) or a biotin–avidin complex, was placed on the sections for 1 hr. After washing, sections were coverslipped in Aquamount.

Immunofluorescent Colocalization of MyHC Isoforms and NCAM

Once injection sites containing donor myoblasts were identified based on X-gal immunohistochemistry, serial sections were ana-

FIG. 4. Comparison of MyHC and NCAM expression in homotypic fibers 28 days after injection into the white gastrocnemius muscle of adult rats. Serial sections were analyzed for β-galactosidase using X-gal histochemistry (A) or for MyHC/NCAM coexpression using immunofluorescent colocalization (B, C). NCAM was detected with a rabbit polyclonal antibody using a fluorescein-conjugated secondary antibody. Mabs specific for neonatal/adult fast MyHC (B, MY-32) or all MyHCs except embryonic and IIX (C, BF.35) were detected using a rhodamine-conjugated secondary antibody. Areas where rhodamine and fluorescein labels are colocalized appear yellow. Colocalization reveals two myotubes (◀) that coexpress NCAM with MY-32 (yellow; B), but not BF.35 (green; C). The absence of BF.35 reactivity in these myotubes indicates that they are coexpressing IIX MyHC with NCAM. This suggests that innervation is not essential for the expression of IIX MyHC in L6BAG-A4-derived homotypic fibers. The homotypic myotube in the upper left of each panel (A) stains with MY-32 (B) but not BF-F35 (C), indicating that it expresses exclusively IIX MyHC. Since it is negative for NCAM, this is an example of an innervated homotypic fiber which continues to express IIX MyHC. Bar, 30 μm.

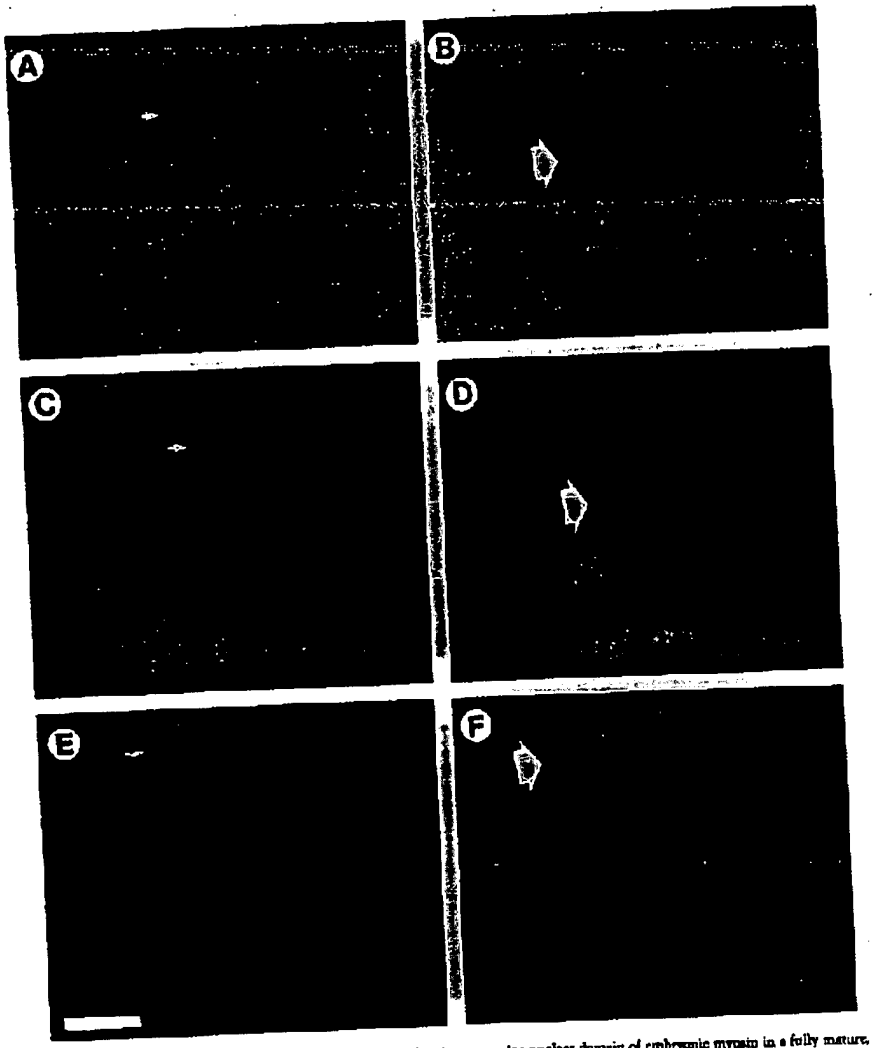


FIG. 5. Rat tibialis anterior muscle 2 weeks after injection showing a putative nuclear domain of embryonic myosin in a fully mature, heterotypic muscle fiber viewed at low (A, C, E) or high (B, D, F) magnification. Serial sections were characterized for X-gal staining (A, B) or MyHC expression using ABC-fluorescence using Mabs against embryonic MyHC (Mab 47A; C and D) or neonatal MyHC (NN6; E and F), and primary antibodies were identified with a rhodamine-conjugated secondary antibody. X-gal staining is fairly uniform (A and B) and shows donor cell fusion to two adjacent fibers (▶). One of the fibers maintains some degree of embryonic MyHC expression localized mainly to the periphery of the fiber (C and D), with some lighter staining throughout the rest of the fiber. The absence of neonatal MyHC (E and F), along with peripheral nucleation revealed by Hoescht dye staining (E) suggests that this fiber is fully mature and under normal circumstances would not express embryonic MyHC. Bar, 100 μm (A, C, E) or 40 μm (B, D, F).

lyzed for (a) the MyHC phenotype of fibers containing donor nuclei, and (b) the presence of NCAM molecules along the surface of the fibers. These sections were fixed with 90% methanol for 6 min at −20°C and then blocked for 30 min with 10% goat serum in PBS at 37°C. In the case of colabeling with mouse monoclonal antibodies, sequential incubation of the primary antibodies (and the secondary antibodies used in their detection) was carried out. Slides were first incubated for 1 hr at RT in IgG_{2A} antibodies (47A and BHB) followed by several rinses of PBS and a 1-hr incubation at RT with fluorescein-conjugated RAM IgG_{2A} (ICN Biomedicals Canada Ltd, Montreal, Quebec), diluted to 1/50 in PBS containing 0.1% bovine serum albumin (BSA). After several rinses with PBS, slides were incubated for 1 hr at RT with a second primary antibody which was specific for IgG_1 antibodies (like MY-32 and BF-35). These antibodies were detected using a rhodamine-conjugated sheep anti-mouse (SAM) IgG_1 (diluted to 1/50 in PBS-BSA). Slides were coverslipped with a 50% glycerol solution in PBS containing 5% paraphenyldiamine and 0.5% Hoescht dye.

To characterize the innervation status of fibers containing donor nuclei, a rabbit polyclonal antibody that recognizes all forms of NCAM (kindly provided by Dr. Geneviève Rougon, CNRS, Marseilles, France; Rougon and Marshak, 1986) was used in conjunction with the various mouse monoclonal antibodies. Since the NCAM antibody is a rabbit polyclonal antibody and the MyHC-specific antibodies are mouse monoclonal antibodies, both primary antibodies were incubated simultaneously. Following incubation in the primary antibodies for 1 hr at RT, sections were rinsed several times with PBS and incubated in a 1:50 dilution of both fluorescein (FITC)-conjugated goat anti-rabbit and rhodamine (RITC)-conjugated rat anti-mouse (RAM) IgG secondary antibodies (ICN Biomedicals Canada Ltd, Mississauga, Ontario) in PBS-BSA for 1 hr at RT. Sections were rinsed several times with PBS and then coverslipped as described above.

Determination of Fiber Types Based on Myosin Heavy Chain Expression

Following ABC-AP immunolocalizations using MyHC-specific Mabs, sections were analyzed for the number of host/donor heterotypic fibers (which stained for X-gal and showed characteristics typical of mature fibers) and host-derived mature fibers. To ensure accurate counting, only fibers clearly belonging to one group or another were scored. Scoring of host-derived fibers was limited to the area immediately adjacent to the injection site so that regional differences in the muscle would be minimized. To provide accurate counts, specific muscle fascicles were analyzed in each serial section. The number of positive fibers within these areas was determined after labeling with 212F (IIB/IIX), 4A.74 (IIA), SC.71 (IIA/IIX), BF.35 (all MyHCs except embryonic and IIX), BF.F3 (IIB), and 10D10 (slow). Fibers were then classified as types I, IIA, IIA/IIX, IIX, IIB/IIX, or IIB. To determine the number of fibers belonging to each group the following equations were used: (A) Total = No. of X-gal positive fibers; (B) I (slow) = No. of 10D10 positive fibers; (C) IIA = Total No. of fibers (A) − No. of 212F-positive fibers = No. of Type I fibers (B); (D) IIA/IIX = No. of SC.71 fibers − No. of Type IIA fibers (C); (E) IIX = No. of 212F-positive fibers − No. of BF.F3-positive fibers − No. of IIA/IIX fibers (D).

Since 212F recognizes both IIX and IIB, it was impossible to accurately determine the number of IIB/IIX fibers. However, Mab 4A.74 has been shown to cross-react with IIX MyHC at higher levels of expression. Therefore, it was possible to obtain a lower limit to the number of fibers containing both IIX and IIB MyHC using the equation:

$$\text{IIB/IIX} = \text{No. of 4A.74-positive fibers} - \text{No. of IIA fibers} - \text{No. of IIA/IIX fibers} - \text{No. of IIX fibers}.$$

Therefore, the number of IIB fibers could be determined by:

$$\text{IIB} = \text{No. of BF.F3-positive fibers} - \text{No. of IIB/IIX fibers}.$$

From these equations the percentage of fibers belonging to each class was determined for regions of the muscle within and outside of the injection sites.

Photography

All images were captured using a Zeiss microscope and the computer software program Northern Exposure. Figures were produced using a Phaser 440 Tektronic dye sublimation printer.

RESULTS

Characterization of L6BAG-A4-Derived Heterotypic Fibers after Injection into Regenerating Adult Muscles

To study the effects of various environments on the maintenance of the L6 phenotype, several different muscles were targeted for injection. The lateral (white) portion of the gastrocnemius is composed predominantly of IIB fibers. The tibialis anterior and plantaris muscles represent typically mixed muscles containing all adult fiber types while the red region of the gastrocnemius and the soleus muscle represent typically slow muscles (Armstrong and Phelps, 1984). These last two muscles contain large numbers of slow and IIA fibers and small numbers of IIX fibers and therefore represent muscle environments in which maintenance of the L6 phenotype may be most challenged.

Following injection, L6 myoblasts formed both homotypic and heterotypic fibers. Homotypic fibers result from the fusion of donor cells with each other to form new myo-

tubes which show intense staining with X-gal, indicating high levels of β-gal activity, and centrally located nuclei characteristic of regenerating fibers (Benoit and Belt, 1970). These myotubes are usually located outside muscle fascicles, often grouped in small clusters at the periphery of the muscle bed. Heterotypic fibers, which result from the fusion of donor myoblasts with host myoblasts or muscle fibers, show considerably weaker X-gal staining, with peripheral nuclei and a typical polygonal shape in cross-sections.

Homotypic myotubes were evident in the plantaris muscle 7 days after injection with the L6BAC-A4 myoblasts, where peripheral clusters of myotubes outside of the muscle bed were observed embedded in either the perimysium or epimysium surrounding muscle fascicles (Fig. 1A; *). The homotypic myotubes were darkly stained with X-gal and typically had a small, circular shape in cross-section. These myotubes extended for up to several centimeters, but they did not extend along the entire length of the muscle. Upon staining with nuclear dyes, central nucleation was observed (not shown). Sections next to those that contained concentrations of homotypic myotubes were subsequently analyzed using ABC-AP immunolocalization to examine MyHC expression. Based on the fiber-type profile in the area adjacent to the injection site, it appeared that cells were often delivered into areas containing a mixture of different fiber types. All myotubes examined at 7 days after injection showed a positive reaction with 47A, typical of both L6 myotubes *in vitro* (Pin and Merrifield, 1997), and muscle fibers undergoing regeneration (Whalen *et al.*, 1990). These cells showed no reaction with BF.35, indicating that neonatal MyHC was not present. Since other antibodies specific for adult fast and slow MyHCs did not react with these myotubes, embryonic MyHC appears to be the first MyHC expressed in nascent myotubes formed by the fusion of L6 myoblasts *in vivo*.

Examination of homotypic fibers in the plantaris muscle 28 days after injection indicated that the shape, size, and X-gal staining intensity characteristic of homotypic fibers was maintained (Fig. 1E). These fibers were also localized toward the periphery of the muscle bed outside the normal muscle fascicle (*). ABC-AP immunolocalization using MyHC-specific Mabs revealed that, in addition to expression of embryonic MyHC evidenced by 47A staining, there was also a second isoform present that was recognized by 212F in the majority of the homotypic fibers. Negative reaction with BF.F3 indicated that this was fast IIX MyHC typical of L6 expression *in vitro*. Fast IIA MyHC was not expressed in these fibers since neither SC.71 or 4A.74 showed a positive reaction. Homotypic L6BAC-A4 fibers also failed to react to any of the slow Mabs. Thus, the pattern of MyHC expression in homotypic fibers derived from L6BAC-A4 myoblasts *in vivo* was remarkably similar to their MyHC profile *in vitro*, with embryonic and adult fast IIX MyHCs being the only isoforms expressed. However, unlike L6 myotubes *in vitro*, homotypic fibers could be detected at 28 days postinjection in which embryonic MyHC was no longer expressed.

Examination of injection sites in the plantaris muscle 56 days after injection revealed that the size, X-gal staining intensity, and peripheral location of homotypic L6BAC-A4-derived muscle fibers was maintained for the duration of the experiments (Fig. 1I). ABC-AP immunolocalization with the various MyHC-specific Mabs demonstrated that the targeted area of the plantaris was made up almost exclusively of IIB fibers. However, examination of the MyHC profile of the L6 myoblast-derived homotypic fibers still revealed positive reactions for 47A and 212F. Since the myotubes did not stain for BF.F3, one can conclude that Mab 212F is recognizing IIX MyHC. All homotypic fibers which reacted with 212F also reacted with MY-32 (not shown), but there were examples where 47A staining was absent, indicating a transition of MyHC expression from embryonic to fast IIX MyHC in a subset of fibers. Since IIA or slow MyHC-specific Mabs did not recognize donor cell-derived fibers, these homotypic muscle fibers most closely resembled IIX fibers.

Numerous other characterizations of homotypic L6BAC-A4-derived muscle fibers demonstrated similar patterns of expression, regardless of the muscle injected. While embryonic MyHC was the predominant isoform early in differentiation, fast IIX MyHC was upregulated over time and gradually replaced the developmental isoform. These observations are summarized in Table 2.

Characterization of NCAM Expression in Homotypic Fibers and Its Relationship to MyHC Expression

Because the loss of embryonic MyHC in some homotypic fibers was observed in all injection sites, we wanted to examine the role of innervation in this transition. To address this issue, a polyclonal rabbit antibody, specific for all NCAM isoforms, was colocalized with the various MyHC antibodies. NCAM is known to be expressed along the entire surface of myotubes prior to innervation. Upon innervation, NCAM becomes localized exclusively to the motor endplate region (Figarella-Branger *et al.*, 1992; Covault and Sanes, 1985). Therefore, myotubes that are NCAM negative are most likely innervated while those that show punctate staining along the membrane are not innervated.

Injection of L6BAG-A4 myoblasts into the regenerating extensor digitorum longus of adult Wister Furth rats produced an area of myotubes within the perimysium between adjacent muscle fascicles at 1 week postinjection (Fig. 2). X-gal immunohistochemistry revealed that these myotubes expressed high levels of β-gal and had a circular cross-sectional shape, typical of donor-derived myotubes. Immunofluorescent localization of serial sections using an NCAM-specific polyclonal antibody and a monoclonal antibody specific for embryonic MyHC revealed that NCAM and embryonic MyHC were essentially coextensive in these cells. This suggested that donor-derived myotubes were not innervated at this early time after injection. Coreactivity of the two antibodies to muscle fibers outside the injection site indicated that the host fibers were undergoing regeneration, a process that can involve both denervation and coexpression of developmental isoforms. Fluorescent immunolocalization with MY-32 revealed that homotypic myotubes at this time after injection did not express neonatal or adult fast MyHCs. The only colabeling of NCAM and MY-32 occurred in host muscle fibers. This was not surprising since these fibers undergo normal regeneration, in which neonatal MyHC is usually expressed.

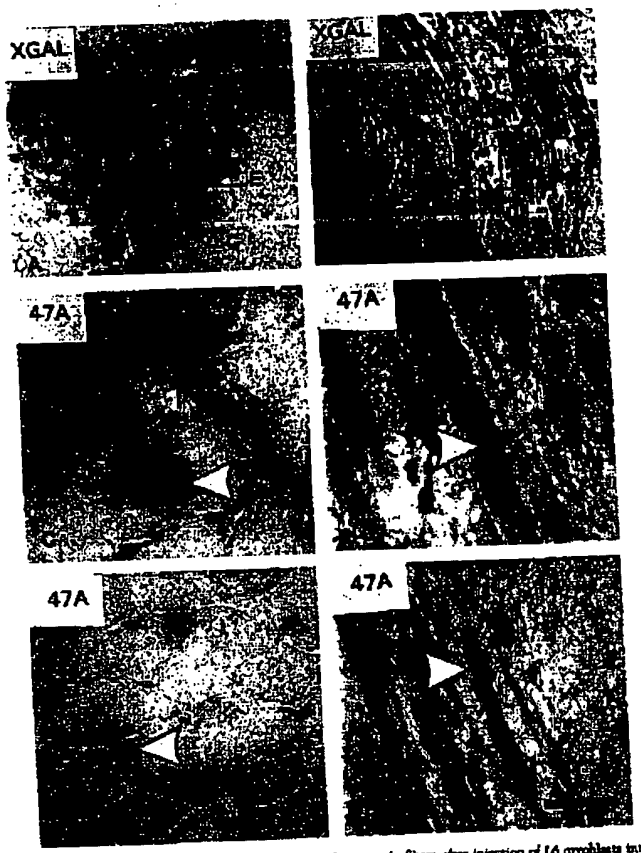
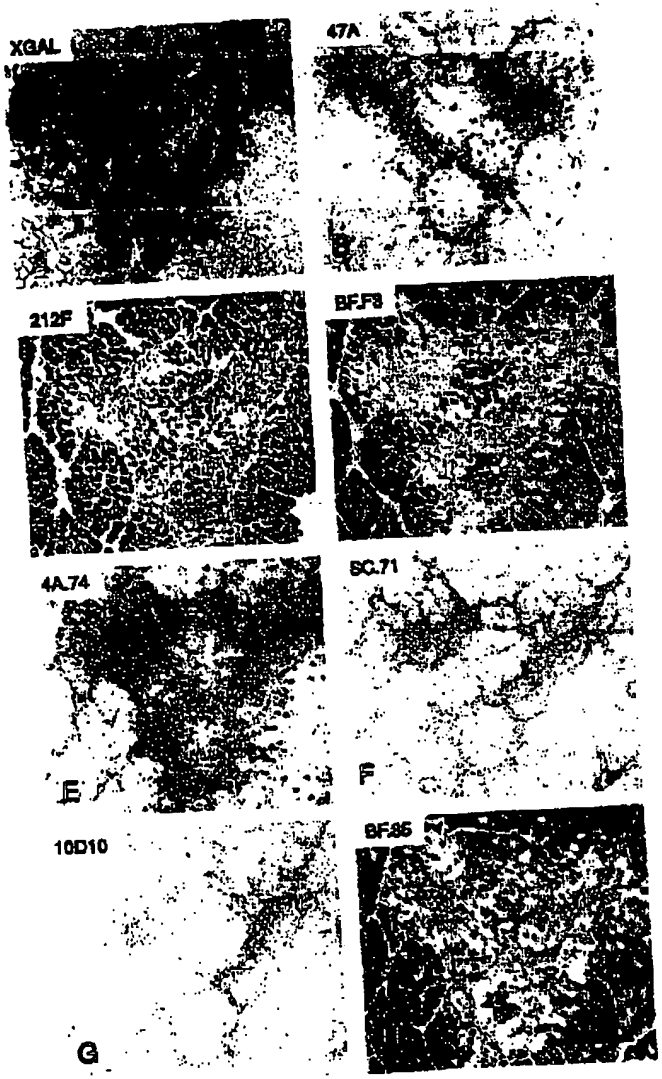


FIG. 6. Nuclear domains of embryonic MyHC persist in adult fast heterotypic fibers after injection of L6 myoblasts into tibialis anterior. X-gal immunohistochemistry revealed large areas of heterotypic fibers in the tibialis anterior at 42 (A) and 56 (B) days after injection. Higher magnification of the boxed areas show ABC-AP immunohistochemistry of 47A revealing nuclear domains of embryonic MyHC within adult fast fibers in the tibialis anterior at 42 (C, E) and 56 (D, F) days after injection. These domains surround single nuclei (▶) and range between 40 and 70 μm in length. These fibers also react with MY-32 (data not shown), indicating that they have an adult fast phenotype. Homotypic fibers between the larger fibers can also be seen (→). Bar, 500 μm for A and B, 40 μm for C and E, and 80 μm for D and F.



To further examine the effects of innervation on the expression of embryonic MyHC, injection sites were analyzed for NCAM and embryonic MyHC expression at 8 weeks after injection into the tibialis anterior muscle (Fig. 3). Immunofluorescent localization of 47A and the NCAM-specific polyclonal antibody revealed homotypic muscle fibers that were positive for NCAM but not 47A, or negative for NCAM and positive for 47A. Therefore, at 8 weeks after myoblast injection, there was still a persistence of the embryonic MyHC isoform, even after innervation had occurred. The presence of homotypic muscle fibers that no longer stained for 47A but still stained strongly for NCAM indicates that the down-regulation of embryonic MyHC can precede innervation. Since there was no correlation between NCAM and embryonic MyHC expression, one can conclude that the developmental switch in embryonic MyHC expression occurs independent of innervation and electrical activity.

To determine if more mature forms of MyHC coincided with the onset of innervation, homotypic fibers were analyzed with Mabs MY-32 (which recognizes all fast MyHC isoforms) and BF.35 (which recognizes all MyHC isoforms except IIX and embryonic MyHC) in conjunction with NCAM expression (Fig. 4). Similar to the *in vitro* phenotype of L6 myotubes, homotypic fibers reacted with MY-32 but not BF.35, indicating the presence of the IIX MyHC isoform. Interestingly, NCAM was colocalized in several myotubes, indicating that innervation had still not occurred. This suggests that, like embryonic MyHC, the expression of the fast IIX MyHC isoform is not regulated by innervation. In addition, some homotypic fibers which did become innervated still exhibited a IIX phenotype, suggesting that the expression of other adult MyHCs (such as type I, IIA, or IIB) was not induced by innervation. Combined, these results indicate that the pattern of MyHC expression in L6BAG-A4-derived homotypic fibers is not dependent upon innervation and that the development of the mature muscle fiber phenotype may be governed by an internal control mechanism.

Expression of Embryonic MyHC in Heterotypic Fibers

Many of the injection sites also contained muscle fibers which exhibited varying intensities of X-gal labeling, peripherally located nuclei, and polygonal-shaped cross-sectional areas characteristic of mature muscle. In addition, β-gal expression was not evenly distributed along the length of these fibers, since areas several hundred micrometers away exhibited little or no staining. These fibers were located within the limits of a muscle fascicle, separated from adjacent fibers by a small amount of connective tissue—the endomysium. Based on these criteria, these fibers were judged to be the result of donor myoblast fusion to host myoblasts and/or muscle fibers.

To determine if the *in vitro* phenotype of L6 myoblasts was maintained when donor and host nuclei were present in a common cytoplasm, these heterotypic fibers were first examined for the expression of embryonic MyHC—the predominant MyHC isoform expressed by L6 cells in culture (Wieczorek et al., 1985; Pin and Merrifield, 1997) and in homotypic muscle fibers *in vivo*. Examination of an injected tibialis anterior muscle 2 weeks after myoblast transplantation revealed putative heterotypic fibers up to several hundred micrometers away from the injection site (Fig. 5). Characterization of these fibers using ABC-fluorescent localization with MyHC-specific antibodies demonstrated regionalized expression of embryonic MyHC. These nuclear domains were concentrated around individual nuclei in one area of the fiber and lightly distributed throughout the rest of the cross-sectional area of the fiber. These fibers appeared to be mature since they were not labeled by NN6, which specifically recognizes the neonatal MyHC characteristic of regenerating fibers. Homotypic fibers in the area did not express neonatal MyHC, since they did not react with NN6.

To determine if the expression of embryonic MyHC was transient, injection sites in the tibialis anterior muscle were analyzed at both 6 and 8 weeks after myoblast transplantation (Fig. 6). ABC-AP localization of Mab 47A revealed the persistence of embryonic MyHC in heterotypic fibers at these later time points. Similar analyses on the contralateral limbs failed to detect embryonic MyHC (data not shown). Interestingly, these nuclear domains were only observed in MY-32-positive fibers, suggesting that the regionalized expression of embryonic MyHC may be restricted to fast fiber types. To determine the approximate size of these nuclear domains, longitudinal sections from the tibialis anterior 8 weeks after injection were characterized. When the length of the nuclear domains was determined by measuring the boundaries of the intense staining, they typically extended 20–25 μm in either direction of an individual nucleus. These results suggest that the embryonic MyHC continues to be expressed for up to 56 days postinjection following the incorporation of L6 myoblasts into fast muscle fibers.

Expression of the IIX MyHC Isoform in Heterotypic Fast Muscle Fibers

Although the embryonic MyHC isoform was observed in putative heterotypic fibers throughout the course of the

FIG. 7. Characterization of heterotypic fibers 42 days after injection of L6 myoblasts into the tibialis anterior of adult Wistar Furth rats. Serial sections were either stained for X-gal (A) or analyzed with ABC-AP immunolocalizations for MyHC-specific Mabs for embryonic (47A; B), fast IIB/IIX (212F; C), fast IIB (BF.F3; D), fast IIA (4A.74, which cross-reacts with IIX; E and SC.71; F), slow (10D10; G), and all isoforms except IIX and embryonic (BF.35; H). A large area of heterotypic, X-gal-stained fibers (A) can be seen to react predominantly with 212F (C) and not with BF.35 (H), indicating the presence of a large population of IIX fibers in an area of the muscle that is predominantly IIB in phenotype (see accompanying table). No other Mab stains to the same extent as 212F; however, 4A.74 (E) shows cross-reaction to this area supporting the idea that this is a predominantly IIX area. Bar, 575 μm.

TABLE 3
Fiber Types in Different Muscles after Injection of L6BAG-A4 Myoblasts

Muscle type	Fiber type	Within I.S.	Percentage of total fibers	Outside of I.S.	Percentage of total fibers
Tibialis anterior	IIX	156	47.4	16	8.2
	IIB	102	31	177	90.8
	IIA	0	0	1	0.5
	IIX/IIB	71	21.6	0	0
	IIA/IIX	0	0	0	0
	I	0	0	0	0
Gastrocnemius	IIX	101	59.8	25	13
	IIB	53	31.4	149	77.2
	IIA	3	1.8	18	9.3
	IIX/IIB	8	4.6	0	0
	IIA/IIX	4	2.4	1	0.5
	I	0	0	0	0
Plantaris	IIX	125	58.4	92	40.2
	IIB	13	6.1	n.d.	n.d.
	IIA	22	10.3	n.d.	n.d.
	IIX/IIB	0	0	4	1.7
	IIA/IIX	41	19.2	n.d.	n.d.
	I	12	5.6	n.d.	n.d.
	I/IIX	1	0.4		

Note. n.d., not determined.

experiment, its absence in some X-gal staining fibers suggested that this expression could be transient. Since this isoform is replaced by the IIX MyHC in homotypic fibers, we next examined the expression of IIX MyHC in donor and host-derived fibers. To analyze the pattern of expression of the IIX MyHC in fast heterotypic muscle fibers, ABC-AP immunolocalization was used to characterize injection sites in the tibialis anterior muscle 6 weeks after injection (Fig. 7). The tibialis anterior muscle contains a superficial region which contains predominantly IIB muscle fibers in normal adult rats (Armstrong and Phelps, 1984). X-gal histochemistry reveal injection sites containing large numbers of heterotypic fibers within this region. These fibers were determined to be heterotypic since in cross-sections several hundred micrometers away they were X-gal negative. Characterization with Mabs specific for the various MyHC isoforms demonstrated that a large proportion of these fibers was not recognized by BF.35 (specific for all MyHC isoforms except IIX), but was recognized by 212F (specific for IIB and IIX). This suggested that these fibers expressed predominantly IIX MyHC. Further characterization of this injection site with SC.71 failed to detect IIA MyHC. Interestingly, 4A.74 lightly labeled the majority of these fibers. This confirmed the presence of the IIX isoform, since this Mab has been shown to cross-react with high levels of the IIX MyHC when used in a sensitive assay such as ABC-immunohistochemistry. To determine the myosin composition of each of these fibers, and to compare the relative proportion of different fiber types within the injection site to areas of normal muscle outside the site, specific fascicles containing all X-gal-positive fibers or all X-gal-negative fibers were identified and scored for MyHC expression. This data was used to calculate relative proportions of each fiber type including types IIB, IIB/IIX, and IIX (Table 3). Type I and IIA fibers were not within or adjacent to the graft and did not enter into the calculations. These analyses revealed that 47.4% of the heterotypic fiber stained exclusively for the IIX MyHC, a 5-fold increase from outside the graft. In addition, another 21.6% of the fibers expressed both fast IIB and IIX, a phenomenon observed only rarely (0.5%) outside the graft, or in contralateral limbs (data not shown). Therefore, 69% of the fibers expressed IIX MyHC, almost a 10-fold increase from the proportion of IIX fibers outside the graft. In contrast, the proportion of exclusively IIB fibers dropped from 90.8% in areas surrounding the injection site, to 31% within the site. These results suggest that the L6 nuclei present within these IIB fibers continue to express IIX MyHC.

Similar characterizations of heterotypic fibers were carried out in an area of the gastrocnemius that contained all fast fiber types (Table 3). Characterization of the muscle with X-gal immunohistochemistry revealed the presence of a large injection site that contained many heterotypic fibers (not shown). ABC-AP immunolocalization with the various MyHC-specific Mabs revealed that these fibers were uniformly recognized by Mab 212F. Further examination indicated that these fibers expressed the IIX MyHC isoform, since neither BF.F3 or BF.35 labeled the majority of the fibers. Evaluation of the MyHC expression within these fiber revealed that almost 60% of the fibers expressed

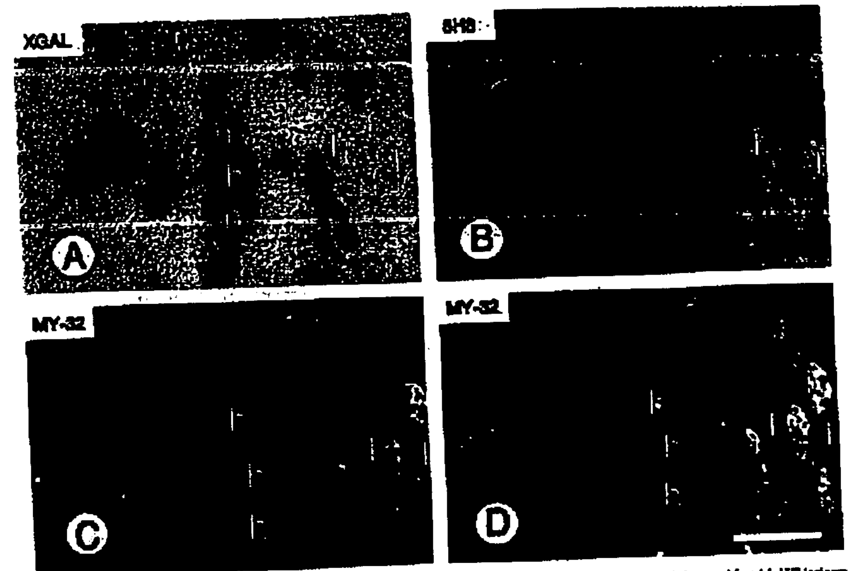


FIG. 8. MyHC expression in the red gastrocnemius muscle 4 weeks after injection showing coexpression of slow and fast MyHC isoforms in β-gal-positive fibers. Serial sections from the injection site were analyzed for β-galactosidase expression using X-gal substrate (A) or with immunofluorescence against slow (8H8, B) or neonatal/adult fast (MY-32, C, D) MyHC. Primary antibodies were recognized by isotype-specific secondary antibodies conjugated to fluorescein (8H8) or rhodamine (MY-32). After 4 weeks, both homotypic (h) and heterotypic (*) fibers can be seen based on morphology and the intensity of X-gal staining (A). Immunofluorescence reveals that the majority of homotypic fibers express adult fast MyHC (C, D) and not slow (B). The heterotypic fibers show coexpression of both adult fast and slow MyHCs (compare B and D), while normal host muscle fibers express only one of the two isoforms. Host type I fibers which express only slow MyHC are indicated by the letter I. Expression of the adult fast isoform is regionalized since sections 70 μm away did not show this pattern of staining of MY-32 staining (C). Bar, 33 μm.

exclusively IIX MyHC and that another 7% expressed the IIX isoform in conjunction with another fast isoform. Therefore, the total proportion of IIX expressing heterotypic fibers was 66.8%—a fourfold increase over regions outside of the graft. In contrast, the proportion of fibers expressing exclusively IIA MyHC dropped from 9.3 to 1.8%, while the proportion of pure IIB fibers decreased from 77.2 to 31.4%. These numbers indicate that the potential of L6BAG-A4 nuclei to express IIX MyHC is maintained following the incorporation of L6 myoblasts into fast muscle fibers of the host.

Expression of the IIX MyHC Isoform in Heterotypic Slow Muscle Fibers

The maintenance of IIX MyHC expression in heterotypic fibers in fast muscles suggested that environmental factors did not affect the expression of the L6BAG-A4 nuclei. To determine if the L6 BAG-A4 *in vitro* phenotype would be maintained in a typically slow environment, myoblasts were injected into the medial gastrocnemius, plantaris, and soleus muscles. Analysis of an injection site in the red gastrocnemius at 4 weeks postinfection revealed a small group of X-gal-positive fibers at the periphery of the muscle (Fig. 8). The light intensity of X-gal labeling coupled with peripherally located nuclei indicated that these were heterotypic fibers containing both host and donor nuclei. Fluorescent localization of MyHCs within the graft revealed that all of the observed heterotypic fibers were recognized by Mabs specific for fast (MY-32) and slow (8H8) MyHCs. The MY-32 antibody was used in place of the 212F antibody since it allowed better sensitivity for detecting the IIX isoform when using indirect immunohistochemistry. All of the fibers adjacent to the injection site reacted with either MY-

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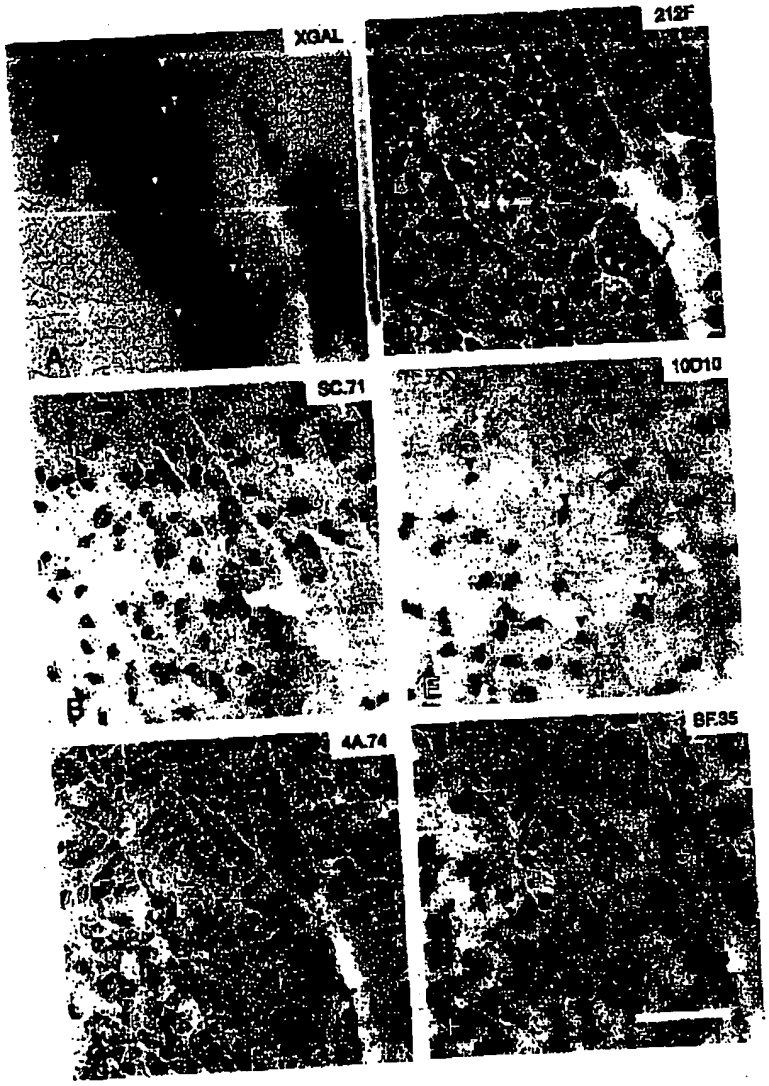

32 or 3H8 but not both, similar to fibers in the contralateral limbs. No labeling was observed when the injection site was characterized with 4A.74, NN6, or 47A (data not shown), indicating that the fast isoform was not IIA or neonatal MyHC and that embryonic MyHC was not present at this time. Characterization of the injection site 100 μm in either direction failed to detect the fast isoform, suggesting that this MyHC was localized to a specific region within the fiber, presumably where fusion of the L6BAG-A4 myoblasts had occurred.

To examine whether the expression of the IIX MyHC isoform was maintained in heterotypic fibers expressing slow MyHC, putative heterotypic fibers were examined at 8 weeks after injection. In order to get a large sample size, injection sites within the plantaris muscle were examined since this muscle contains a mixture of different fiber types. Analysis with X-gal immunohistochemistry, revealed a large area of heterotypic fibers (Fig. 9), which were subsequently characterized using ABC-AP immunolocalization with MyHC-specific Mabs. Unfortunately, the percentage of IIX/IIB fibers could not be determined since the 4A.74 Mab did not cross-react with the fibers expressing IIX MyHC. However, it was still possible to calculate the proportions of types I, IIA, IIA/IIX, and IIX fibers (Table 3). Even though the percentage of exclusively IIX fibers did not show a large increase within the injection site, 19.2% of the fibers expressed both IIX and IIA MyHCs. The total number of fibers which expressed IIX MyHC alone or in combination with IIA was 77.6%, an increase of 35.7% over the area outside the injection site.

Upon examination of the injection site with 212F and 10D10, heterotypic fibers coexpressing slow and fast MyHC were not detected. These findings were confirmed by similar observations in the soleus and red gastrocnemius muscles at 8 weeks after injection (data not shown). In addition, slow muscle fibers analyzed over several hundred micrometers using serial sections revealed no aberrant fast IIX MyHC accumulations. Although it is possible that putative nuclear domains of IIX or embryonic MyHC could have been overlooked, it seems likely that the fusion of L6 myoblasts to slow fibers resulted in the down-regulation of IIX MyHC by L6 nuclei at 8 weeks postinjection.

DISCUSSION

The introduction of L6BAG-A4 myoblasts into a regenerating muscle environment allows these myoblasts to fuse with each other or with host satellite cells and regenerating muscle fibers to form both homotypic and heterotypic muscle fibers. Homotypic fibers are usually formed by the large population of cells which remain at the periphery of the muscles or between muscle fascicles. Since these myotubes maintain the characteristic IIX MyHC expression of L6 cells and down-regulate embryonic MyHC expression, these results support the hypothesis that L6 myoblasts display the unique potential of forming exclusively fast IIX myotubes both *in vitro* and *in vivo*. The observation that myoblasts maintain their characteristic *in vitro* MyHC profile after injection into regenerating muscle supports work recently done in an avian model (DiMario *et al.*, 1993; DiMario and Stockdale, 1995), in which primary quail myoblasts of either a fast or fast/slow lineage were injected into fetal chick muscles. These injections resulted in the formation of homotypic myotubes which expressed either fast or fast/slow MyHCs in all muscle environments examined. However, since these myotubes were only followed 10 days *in vivo*, these experiments did not address the possibility of any long-term effects of the environment in general or innervation in particular. The observation that L6BAG-A4-derived homotypic fibers become innervated *in vivo*, and that this innervation does not effect the final phenotype of the myotubes, therefore extends the observations previously made in birds. In addition, this is the first demonstration of a fiber-type-specific myoblast cell lineage in mammals.

The innervation of these fibers is not suprising considering denervated myotubes have been shown to express increased levels of neurotrophic factors (Oppenheim *et al.*, 1993) and higher levels of NCAM (Covault and Sanes, 1985), two factors known to play a role in muscle/nerve interaction and final innervation (Landmesser *et al.*, 1988). The observation that these homotypic myotubes become innervated is consistent with experiments that were carried out by Wernig *et al.* (1991), in which putative homotypic fibers reportedly became innervated starting at 4 weeks after injection of cloned neonatal mouse myoblasts into regenerating mouse muscle. Although this group noticed that a fast fiber phenotype predominated early after injection, they also observed a transition to Type I fibers, suggesting that environmental influences may eventually control the phenotype of the myoblasts. These experiments were limited, however, by the fact that the myoblasts used were not characterized *in vitro*, and that the assays used (acid ATPase) were not sensitive enough to delineate between various subtypes of fast fibers. Although we observed innervated homotypic fibers which continued to express exclusively IIX MyHC, the

FIG. 9. Characterization of heterotypic fiber 56 days after injection of L6 myoblasts into the plantaris of Wistar Furth rats. Serial sections were either stained for X-gal (A) or analyzed with ABC-AP immunolocalizations for MyHC-specific Mabs for fast IIB/IIX (212F; B), fast IIA (SC.71; C), slow (10D10; D), adult fast IIA (4A.74; E), or all isoforms except IIX and embryonic (BF.35; F) MyHCs. A large area of heterotypic, X-gal-stained fibers (A) can be seen in a mixed muscle containing all fiber types. Although this is a mixed area, it can be seen that the predominant fiber type is fast IIX, both within and outside of the graft site, since BF.35 (F) stains less than half of the fibers. Staining with 10D10 reveals the presence of several slow type fibers within the graft (D), none of these react with 212F, indicating that the adult slow isoform does not colocalize with fast IIB/IIX. Bar, 90 μm.

nature of the motoneurons involved could not be determined with any precision. Since selective innervation by fast motoneurons could have occurred, this approach did not allow us to address the effects of different types of neural influences on MyHC expression by L6 cells.

The presence of donor-derived nuclei within heterotypic fibers did enable us to examine the effects of different types of innervation and cytoplasms on the expression of L6-derived nuclei. When L6 myoblasts fused to typically fast host fibers, they maintained their expression of both embryonic and fast IIX MyHC. The retention of embryonic and/or IIX MyHC expression by donor nuclei incorporated into a vast excess of host sarcoplasm is direct evidence that individual nuclei within a muscle fiber can behave independently, and is in agreement with previous studies by others who examined the selective expression of acetylcholine receptor subunits (Simon *et al.*, 1992) and acetylcholine receptor-LacZ transgenes (Sanes *et al.*, 1991) by subsynaptic nuclei. These accumulations were small (≈ 60 μm) and were usually located at the periphery of the fiber, although in some instances they covered the entire cross-sectional area of the fiber. Previous studies involving myoblast heterokaryons in culture have shown that MyHC isoforms can form nuclear domains around the nucleus of origin (Pin and Merrifield, 1997; Pavlath *et al.*, 1989). This compartmentalization of MyHC in heterotypic fibers is similar to the subsynaptic localization of novel MyHCs induced by ectopic innervation of muscles by foreign neurons (Salviati *et al.*, 1986).

It is possible that these pockets of expression may represent localized areas of regeneration since developmental isoforms are reexpressed upon regeneration of muscle. However, this is unlikely since these accumulations persist throughout the length of the study in histologically mature fibers. The reexpression of embryonic MyHC in the contralateral limb lasts approximately 14 days, which is in agreement with other published reports (D'Albis *et al.*, 1988, 1989). It is also possible that these accumulations represent myoblasts fusing onto mature fibers long after injection. While such fusion events have been shown to occur between satellite cells and undamaged areas of muscle fibers (Robertson *et al.*, 1992), it is unlikely that this accounts for accumulations of embryonic MyHC 56 days after injection, since proliferation of cells after transplantation peaks at 7 days and is finished by 10 days after injection (Roberts *et al.*, 1989). The most likely explanation of these results is that these accumulations represent nuclear domains in which the L6 phenotype is being maintained, overriding external cues such as innervation.

Interestingly, the incorporation of L6 nuclei into slow fibers resulted in only transient expression of the IIX isoform and by 8 weeks, IIX MyHC could no longer be observed in X-gal positive slow fibers. Therefore, the maintenance of such domains was only observed in fast muscles, suggesting that the type of innervation may override the intrinsic L6 program of expression. Other studies involving the injection of either the mouse C_2C_{12} cell line or cloned satellite cells into adult hindlimb muscles (Hughes and Blau, 1992) has revealed similar changes in the myoblast phenotype. Hughes and Blau observed that the injected myoblasts adapted in all cases and that the change was based strictly on the environment in which they were placed. However, both C_2C_{12} myoblasts (MacIntyre and Merrifield, 1997) and cultured satellite cells (Edom *et al.*, 1994) can express both fast and slow MyHCs and may represent a multipotential myoblast stem cell population. Therefore, these donor cell populations appear to be more susceptible to environmental cues than the L6 cell line, which has a limited phenotypic potential in culture.

It is possible that the maintenance of the IIX and embryonic MyHCs is due to a permissive environment found in the fast fiber type populations. Although colocalization of various fast mRNA transcripts has previously been reported in normal adult rat hindlimbs (DeNardi *et al.*, 1993; Campione *et al.*, 1993), coexpression of specifically fast IIX MyHC with slow MyHC isoforms has not been observed, suggesting that some type of restriction may be placed upon slow muscle fibers which prevents the accumulation of IIX MyHC. One potential mechanism that would allow for the internal regulation of IIX MyHC involves changes in the expression of the myogenic regulatory factors (mrfs). It has been postulated that differential expression of the mrfs may be involved in the establishment of slow and fast fiber types, with higher amounts of MyoD existing in fast fibers and higher amounts of myogenin in slow fibers (Hughes *et al.*, 1993). Indeed, differences in mrf expression have been identified in distinct population of myoblasts, which may be related to their developmental potential *in vivo* (Smith *et al.*, 1993; Cusella-DeAngelis *et al.*, 1992; Rudnicki and Jaenisch, 1995). Although L6 myoblasts do not express MyoD (Hinterberger *et al.*, 1991), MRF4 is expressed at the same time that IIX MyHC is first detected, suggesting that IIX MyHC accumulation may be linked in some fashion to MRF4 gene expression. Although the limb muscles of MRF4 knock-out mice have normal distributions of fast and slow fibers (Braun and Arnold, 1995; Zhang *et al.*, 1995; Patapoutian *et al.*, 1995), the expression of IIX MyHC in these animals has not been specifically examined. Even a normal distribution of IIX fibers in these animals would not rule out a role for MRF4 in regulating IIX MyHC gene expression, since the overexpression of myogenin in MRF4 knock-out mice may compensate for the absence of MRF4 (Zhang *et al.*, 1995).

In conclusion, the results suggest that the endogenous "default" program of the L6 myoblasts which is expressed *in vitro* is maintained in homotypic and heterotypic fast muscle fibers *in vivo*. Although environmental influences may be capable of repressing the expression of IIX MyHC in slow muscle fibers, the factors responsible for mediating this effect are currently unknown. It is also not clear whether the incorporation of L6 myoblasts into denervated slow fibers would be sufficient to induce the expression of slow MyHC in L6-derived nuclei *in vivo*. Experiments are currently underway in our laboratory to address this question.

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Both Myoblast Lineage and Innervation Determine Fiber Type and Are Required for Expression of the Slow Myosin Heavy Chain 2 Gene

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Skeletal muscle fibers express members of the myosin heavy chain (MyHC) gene family in a fiber-type-specific manner. In avian skeletal muscle it is the expression of the slow MyHC isoforms that most clearly distinguishes slow- from fast-contracting fiber types. Two hypotheses have been proposed to explain fiber-type-specific expression of distinct MyHC genes during development—an intrinsic mechanism based on the formation of different myogenic lineage(s) and an extrinsic, innervation-dependent mechanism. We developed a cell culture model system in which both mechanisms were evaluated during fetal muscle development. Myoblasts isolated from prospective fast (pectoralis major) or slow (medial adductor) fetal chick muscles formed muscle fibers in cell culture, none of which expressed slow MyHC genes. By contrast, when muscle fibers formed from myoblasts derived from the slow muscle were cocultured with neural tube, the muscle fibers expressed a slow MyHC gene, while muscle fibers formed from myoblasts of fast muscle origin continued to express only fast MyHC. Motor endplates formed on the fibers derived from myoblasts of both fast and slow muscle origin in cocultures, and slow MyHC gene expression did not occur when neuromuscular transmission or depolarization was blocked. We have cloned the slow MyHC gene that is expressed in response to innervation and identified it as the slow MyHC 2 gene, the predominant adult slow isoform. cDNAs encoding portions of the three slow myosin heavy chain genes (MyHC1, slow MyHC 2, and slow MyHC 3) were isolated. Only slow MyHC 2 mRNA was demonstrated to be abundant in the cocultures of neural tube and muscle fibers derived from myoblasts of slow muscle origin. Thus, expression of the slow MyHC 2 gene in this *in vitro* system indicates that formation of slow muscle fiber types is dependent on both myoblast lineage (intrinsic mechanisms) and innervation (extrinsic mechanisms), and suggests neither mechanism alone is sufficient to explain formation of muscle fibers of different types during fetal development. © 1997 Academic Press

INTRODUCTION

Vertebrate skeletal muscles are composed of muscle fibers formed from muscle precursor cells, called myoblasts. Within each muscle fiber a number of muscle-specific contractile proteins are synthesized from families of genes encoding multiple protein isoforms. The combinations of isoform genes expressed in particular muscle fibers are large and diverse (Staron and Pette, 1987) giving rise to a multiplicity of muscle fiber phenotypes with unique repertoires of muscle-specific proteins and associated physiological characteristics. In particular, expression of members of the myosin heavy chain (MyHC) multigene family defines fiber type identity and significantly affects fiber contractile properties via myosin ATPase activity (Reiser *et al.*, 1988a,b). In broad terms, fibers are classified as fast, fast/slow (mixed), or slow depending on the presence of MyHC isoforms with fast and/or slow ATPase activities. A central issue in myogenesis is what determines the formation of different muscle fiber types (Stockdale, 1997).

Members of both fast and slow MyHC gene subfamilies are expressed in developmental and tissue-specific patterns. Transitions in fast MyHC isoforms occur in nearly all avian skeletal muscle fibers. These transitions typically involve successive expression of embryonic and then neonatal and, finally, adult fast MyHC genes (Whalen *et al.*, 1981; Crow and Stockdale, 1986a; Bandman and Bennett, 1988). However, the final fast MyHC genes expressed in adult muscle

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